American Journal of Pathology, Vol. 142, No. 5, May 1993

Regulation of the Differentiation of Diploid and Some Aneuploid Rat Liver Epithelial (Stemlike) Cells by the Hepatic Microenvironment

William B. Coleman,* Anne E. Wennerberg,† Gary J. Smith,* and Joe W. Grisham*

From the Department of Pathology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and the Department of Pathology, University of Pittsburgh,† Pittsburgh, Pennsylvania*

Following intrahepatic transplantation in adult syngeneic Fischer 344 rats, diploid cultured rat liver epithelial cells (WB-F344), modified to carry the* Escherichia coli *β-galactosidase reporter gene and/or the fluorescent membrane dye PKH26-GL, integrate into hepatic plates and acquire the size and nuclear structure of mature hepatocytes. Additionally, of two aneuploid, neoplastically transformed derivatives of WB-F344 cells, both of which produce aggressively growing tumors when transplanted subcutaneously, cells of one line (GN6TF) do not produce tumors in the liver but integrate into hepatic plates and morphologically differentiate. The other transformed line (GP7TB) retains tumorigenicity in the liver, but cells in the intrahepatic tumors are more differentiated morphologically than are tumors at subcutaneous sites. These results suggest that WB-F344 cells are stemlike cells for hepatocytes and that the hepatic microenvironment induces them to incorporate into hepatic plates and differentiate. Our results also suggest that the hepatic microenvironment regulates the differentiation of some neoplastically transformed hepatic stemlike cells, thereby eliminating or reducing their tumorigenic potential. (Am J Pathol 1993, 142:1373–1382)

The liver is composed of two major types of differentiated epithelial cells: hepatocytes, located in the hepatic parenchymal plates, and biliary epithelial cells located in the bile ducts. Both of these differentiated lineages are derived embryonically from a common founder cell, the hepatoblast, a derivative of the endoderm of the ventral foregut.[1–3] Although both hepatocytes and biliary duct epithelial cells in the adult rodent can proliferate to meet replacement demands of cells lost from the respective differentiated populations,[4] evidence suggests that the livers of adult rats also contain undifferentiated epithelial cells that have some of the major properties expected of stem cells for both lineages.[5,6] Small, morphologically and functionally simple epithelial cells can be isolated directly from heterogeneous suspensions of rat liver cells prepared by enzymatic perfusion of the liver, under conditions that exclude differentiated hepatocytes.[7–9] Studies on the reaction of rat liver to a variety of injuries, including some carcinogenic regimens, have led to the hypothesis that facultative liver stem cells may be activated to proliferate and that their progeny may participate in the development of liver cancer and other pathological responses.[5–14] At least some of these pathological responses are mediated through the proliferation and subsequent differentiation of so-called oval cells,[10] which may represent activated liver stem cells.[10–14] Oval cells proliferating after carcinogenic or noncarcinogenic liver injury have been shown experimentally to differentiate into hepatocytes.[15–20] Nonetheless, the existence of a stem cell for hepatocytes is still controversial.[5,6]

We have previously reported the establishment of a cloned diploid epithelial cell line (designated WB-F344) from the liver of a young adult male Fischer 344 rat, shown that the cells were not derived from differentiated hepatocytes, and characterized some of their phenotypic properties.[21] Although WB-F344 cells share some major phenotypic properties with both hepatocytes and bile duct epithelial cells, their overall phenotype differs distinctively from either differentiated cell type but resembles that of some lines

Supported by NIH grant CA 29323. WBC and AEW were supported, in part, by NIH training grant T32 ES 07017.

Accepted for publication November 18, 1992.

Address reprint requests to Dr. Joe W. Grisham, Kenan Professor and Chair, Department of Pathology, CB 7525, University of North Carolina, Chapel Hill, North Carolina, 27599.

of oval cells.[21,22] Neoplastic transformation of WB-F344 rat liver epithelial cells *in vitro* yields cell lines which display various transformed properties, including tumorigenicity.[23–25] When transplanted into subcutaneous and intraperitoneal sites of syngeneic rats, neoplastically transformed WB-F344 cells produce a spectrum of tumors, including highly differentiated hepatocellular carcinomas, hepatoblastomas, and adenocarcinomas (ductlike),[26] demonstrating their potential to differentiate along both hepatic epithelial lineages.

In this study, we have examined directly the ability of WB-F344 rat liver epithelial cells[21] and of selected tumorigenic progeny of the WB-F344 cell line[23–25] to differentiate morphologically into hepatocytes under the influence of the microenvironment of the liver. The results demonstrate that WB-F344 cells give rise to hepatocytes of the liver parenchyma *in vivo* following transplantation. In addition, the present study shows that the microenvironment of the liver parenchyma exerts a regulating influence that suppresses or reduces the tumorigenicity of some highly tumorigenic variants of WB-F344 cells, perhaps by inducing terminal hepatocytic differentiation.

Materials and Methods

Cell Lines and Culture

The cell lines utilized in these studies were derived from a normal nonhepatocytic rat liver epithelial cell line termed WB-F344.[21] Phenotypically distinct γ-glutamyl transpeptidase-positive and negative cell lines were established from a tumorigenic population of WB-F344 cells transformed *in vitro* by 11 consecutive brief treatments with 5 μg/ml N-methyl-N′-nitro-N-nitrosoguanidine.[22–25] Tumors which formed in syngeneic animals after transplantation of transformed cell lines gave rise to tumor cell lines that expressed altered phenotypic properties and produced tumors in 100% of animals after a short latency period.[27] Two of the tumorigenic cell lines isolated from these tumors, GN6TF (derived from a tumor that resulted following transplantation of cloned line GN6) and GP7TB (derived from a tumor that resulted following transplantation of cloned line GP7), were utilized in these studies. These tumor cell lines are aneuploid[27] and carry similar marker chromosomes (S Steadman, LW Lee, GJ Smith, JW Grisham, unpublished observation). The WB-F344 cells and tumor cell lines were maintained in Richter's improved minimal essential medium with zinc option (Imemzo) supplemented with insulin, as modified[28] from Richter et al.[29]

Production of BAG2 Retrovirus and Infection

The CRE BAG2 cell line (American Type Culture Collection #CRL 1858) is a retrovirus packaging mutant[30] that produces an infectious defective retrovirus[31] encoding the Tn5 neomycin resistance gene and the *Escherichia coli* β-galactosidase gene of the Lac operon.[32] Conditioned medium containing infectious BAG2-retrovirus was collected from cultures of CRE BAG2 producer cells that had been held at confluence for 4 days. Infectious medium was passed through a 0.45-μ filter to remove cellular debris and used directly. Assays for helper virus using National Institutes of Health 3T3 cells were performed as recommended by the American Type Culture Collection. No helper virus activity was detected in any of the viral preparations used for these studies.

Infection of WB-F344 cells and tumorigenic progeny with the BAG2-retrovirus was performed essentially as described.[32] Recipient cell lines (WB-F344, GN6TF, and GP7TB) were infected in the presence of 8 μg/ml 1,5-dimethyl-1,5-diazaundecamethylene polymethylbromide (polybrene) for 4 to 6 hours at 37 C. Infected cells were plated with Richter's Imemzo medium[28,29] containing 800 μg/ml G418 (neomycin sulfate) 24 hours after infection. Cells were re-fed every 3 days with fresh medium containing G418 until colonies of G418-resistant cells were obtained. Colonies exhibiting G418 resistance were isolated using cloning rings and established as individual cell lines. No deleterious effects on the ability of these cells to thrive in culture were observed following infection with the BAG2-retrovirus.

Cell Staining with PKH26-GL

The fluorescent membrane dye PKH26-GL[33] was utilized in some studies as an alternative marker for transplanted cells. This membrane dye forms a stable marker for transplanted cells with no transfer of the dye from labeled cells into the adjacent nonlabeled cells following transplantation.[33] Labeling conditions employed were essentially as described by the manufacturer (Zynaxis Cell Sciences, Malvern, PA). Briefly, trypsinized cells were washed twice in serum-free medium, resuspended to a density of 2×10^7 cells/ml in Diluent C (provided by Zynaxis) and mixed with an equal volume of the PHK26-GL dye in Diluent C to give a final dye concentration of 10 μmol/L. Labeling was carried out at room temperature for 10 minutes. The labeling reaction was terminated by the addition of an equal vol-

ume of fetal bovine serum. Labeled cells were diluted with culture medium containing serum and washed once. These labeling conditions significantly diminished cell viability (by ~20 to 30%). Therefore, cells were routinely replated after labeling with the fluorescent dye and cultured overnight to allow them to recover. Cell preparations used for transplantation had viabilities greater than 95%.

Cell Transplantation

Five independent transplantation series were carried out using BAG2-WB cells. The BAG2-WB cells were introduced into the liver tissue by direct transcapsular injection into a liver lobe (n = 32 rats) or by transplantation into the spleen (n = 8 rats). Fifteen animals received BAG2-WB cells that had been labeled with PKH26-GL[33] to provide a secondary marker for transplanted cells (as described above). In two of these injection series, individual rats were subjected to partial hepatectomy[34] in conjunction with the transplantation of cells (n = 8). Partially hepatectomized animals received BAG2-WB cells by direct injection into a lobe of the residual liver or into the spleen following the hepatic surgery. Groups of control rats included in each of the transplantation series consisted of normal controls (no cells transplanted, no surgery performed) and transplantation controls (transplanted with the parental cell line). Specifically, 11 control rats received transplants of parental WB-F344 cells (lacking the reporter gene construct), and another 18 normal control rats provided liver tissue that had not been transplanted with cells. The BAG2-GN6TF and BAG2-GP7TB cells were transplanted into the livers of recipient rats in the same manner. A total of 12 experimental animals and 10 transplantation control animals were utilized for the transplantation series with transformed cell lines.

Cells intended for transplantation (BAG2-WB, BAG2-GN6TF, BAG2-GP7TB, or the parental lines) were trypsinized, washed in three changes of ice-cold Thilly's buffered salt solution to remove traces of serum, resuspended in ice-cold Thilly's buffered salt solution at a concentration of 5×10^6 cells/0.2 ml, and held on ice until used. Cells in a volume of 200 μl were injected with a tuberculin syringe directly into the median liver lobe or into the dorsal tip of the spleen of male rats anesthetized lightly with ether. When needed, hemostasis was maintained by applying a Gelfoam sponge to the site of injection. No loss of cell viability was evident upon culture of cells following their maintenance on ice during injection procedures (1 to 2 hours). Studies involving the use of animals were carried out in accordance with federal and institutional guidelines put forth by the National Institutes of Health and the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

β-Galactosidase Histochemistry

Clonal cell lines isolated from G418-resistant colonies following infection of the parental rat liver epithelial cell lines with the BAG2-retrovirus were characterized for β-galactosidase expression *in vitro* using 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal). Cell monolayers were fixed in an ice-cold solution of 1% glutaraldehyde containing 100 mmol/L $NaPO_4$ (pH 7.0) and 1 mmol/L $MgCl_2$ for 15 minutes, rinsed briefly in 100 mmol/L $NaPO_4$ (pH 7.0), and incubated for 4 to 16 hours at 37 C in an X-gal substrate modified from that of Sanes et al[35] containing 25 mmol/L $NaPO_4$ (pH 7.0), 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 3.3 mmol/L $K_4Fe(CN)_6 \cdot 3H_2O$, 3.3 mmol/L $K_3Fe(CN)_6$, and 0.2 mg/ml X-gal. The X-gal was dissolved in N,N′-dimethylformamide at 20 mg/ml, and then diluted into the substrate reaction mixture. Liver cryosections (8 to 10 μ) containing transplanted BAG2-WB, BAG2-GN6TF or BAG2-GP7TB cells were fixed with glutaraldehyde, and β-galactosidase histochemistry[35] was performed as described for cell monolayers.

We observed that the endogenous lysosomal β-galactosidases of rat cells (especially in splenic macrophages and to a much lesser extent in Kupffer cells and rare hepatocytes) that display acid pH optimas[36–39] retain sufficient activity at the neutral pH optimum of the bacterial enzyme to yield appreciable blue chromogen from X-gal. To reduce the possibility that β-galactosidase-positive cells in liver cryosections represent the expression of endogenous activities in host macrophages or hepatocytes, control liver cryosections were incubated in X-gal substrate in parallel with liver cryosections from experimental rats. Groups of control rats consisted of both normal adult rats and adult rats transplanted with parental cell lines lacking the reporter gene construct (as described above). The conditions routinely used for detection of transplanted cells (<16 hours incubation time in X-gal substrate) minimized the numbers of host cells (hepatocytes or macrophages) yielding discernible β-galactosidase activity in control liver cryosections. In contrast, histochemical identification of transplanted

cells (which carry the *E. coli* β-galactosidase gene) within the spleen was not possible due to the extremely high level of endogenous β-galactosidase activity observed in splenic macrophages.

Polymerase Chain Reaction (PCR)

Synthetic oligodeoxynucleotide primers complementary to the DNA sequences located in the Tn5 neomycin resistance gene and the SV40 region of the BAG2-retroviral DNA were used. The 5′ amplification primer (5′-GATCAAGAGACAGGAGGATCGGTTTCGC) is complementary to the coding sequence of the neomycin resistance gene,[40] and the 3′ primer (5′-GGATCCAGACATGATAAGATACATTGATGAG) recognizes the SV40 polyadenylation signal.[41] Thirty-five cycles of amplification with the *Thermus aquaticus* polymerase were performed using 95 C for denaturation, 58 C for primer annealing, and 72 C for elongation. In positive samples, the amplification reaction produced a 1.95-kb DNA fragment. Reactions containing control liver DNA as the template for PCR failed to produce an amplified DNA product.

Results

We established cell lines carrying the *E. coli* β-galactosidase reporter gene and the neomycin resistance gene by infection of WB-F344 cells and cells of two tumor lines (GN6TF and GP7TB) with the BAG2-retroviral construct.[32] Infected cell lines, termed BAG2-WB, BAG2-GN6TF, and BAG2-GP7TB, were selected by their neomycin resistance and by their expression of histochemically detectable[35] levels of β-galactosidase (as shown in Figure 1). Southern analysis of DNA isolated from BAG2-infected cell lines demonstrated that the neomycin resistance gene had been inserted into the host genome (data not shown). Assays for helper virus in successfully infected cell lines demonstrated that the BAG2-cells used in this study produced no infectious BAG2 virus (data not shown).

BAG2-WB cells established from WB-F344 cells were transplanted into livers of adult rats to investigate the fate of these cells within the microenvironment of the liver. For some transplantation experiments, BAG2-WB cells were also labeled with the fluorescent membrane dye PKH26-GL.[33] Rats were sacrificed at various intervals (from 7 to 120 days) following transplantation of BAG2-WB cells, and livers were examined for the presence of β-galactosidase-positive and/or PKH26-GL-positive cells. Examination of livers from rats transplanted with BAG2-WB cells revealed that β-galactosidase-positive cells were incorporated into the hepatic plates among the host hepatocytes (Figure 2, A to F). The presence of β-galactosidase-positive cells within the plates of the hepatic parenchyma was observed at all time points up to 17 weeks following transplantation, the limits of this study. These β-galactosidase-positive cells resembled host hepatocytes in terms of nuclear structure and cell size. Differentiated hepatocytes are more than twice the size of WB-F344 cells[21] and, in contrast to the latter, contain larger, spherical nuclei with prominent nucleoli.[7–9,21] The large size of the β-galactosidase-positive cells and the structure of their large vesicular nuclei suggest that the morphology of the BAG2-WB cells changed in conjunction with their

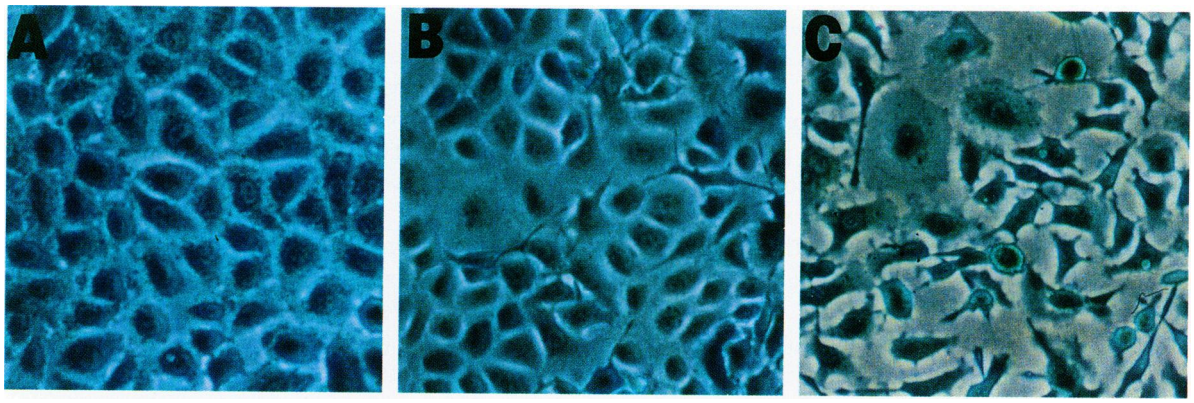

Figure 1. *Expression of histochemically-detectable β-galactosidase activity in BAG2-infected cell lines. Clonal cell lines isolated from G418-resistant colonies following infection of parental cell lines with the BAG2-retrovirus (BAG2-WB, BAG2-GN6TF and BAG2-GP7TB) were characterized for β-galactosidase expression in vitro using X-gal. The parental cells (WB-F344, GN6TF, and GP7TB) in each case did not express any endogenous β-galactosidase activity and therefore did not produce blue chromogen upon incubation with X-gal (data not shown).* **A:** *BAG2-WB20.6 cells,* **B:** *BAG2-GN6TF cells,* **C:** *BAG2-GP7TB cells. (Magnification ~ × 380).*

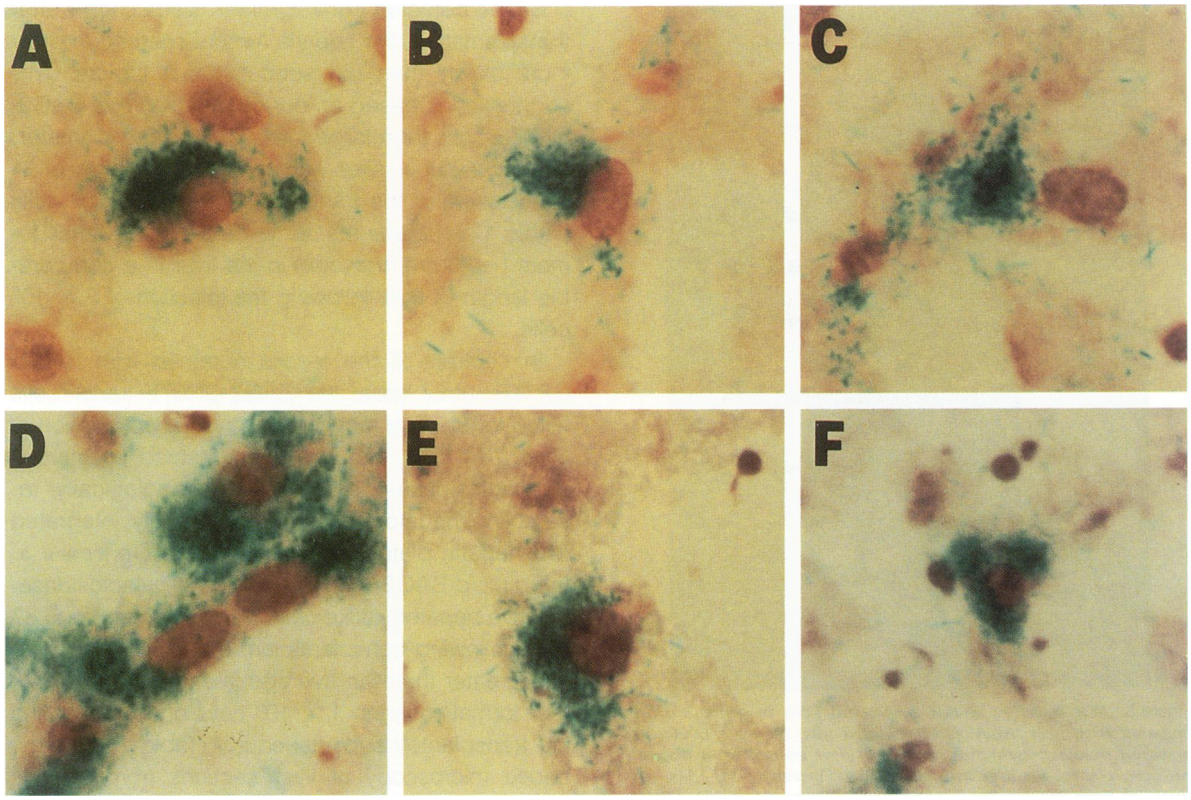

Figure 2. *Cryosections demonstrating the presence of transplanted BAG2-WB cells in the liver parenchyma of transplanted rats. Liver tissue was obtained from rats at various intervals (7 to 120 days) following transplantation with BAG2-WB cells. Freshly isolated tissue was flash frozen in isopentane, cooled in liquid nitrogen, and cryosections prepared. Cryosections (~8 to 10 μ) were fixed briefly with glutaraldehyde, histochemically stained by reaction with an X-gal substrate for 4 to 16 hours, and counterstained in Mayer's hematoxylin solution.* **A,B:** *Liver sections from a rat 30 days following transplantation.* **C,D:** *Liver sections from a rat 50 days following transplantation.* **E,F:** *Liver sections from a rat 87 days following transplantation. (Magnifications:* **A** *to* **E**, ~ ×800; **F**, ~ ×550*).*

incorporation into hepatic plates. Identical results were obtained when BAG2-WB cells were introduced into the liver tissue via injection into the spleen (data not shown) and when transplantation of cells was accompanied by partial hepatectomy (data not shown).

Identification of β-galactosidase-positive cells outside the hepatic plates as transplanted BAG2-WB cells was complicated by the presence of Kupffer cells, which sometimes show β-galactosidase activity histochemically.[42] Hepatocytes in liver cryosections from control rats exhibited negligible background staining for β-galactosidase under the conditions for histochemical detection utilized (as described in Materials and Methods; data not shown). No significant difference was observed in the level of endogenous β-galactosidase activity detected in liver cryosections from normal controls and in those from rats that were transplanted with parental cell lines that lacked the reporter gene (data not shown). The presence of transplanted BAG2-WB cells in the liver parenchyma was verified using the lipophilic fluorescent membrane labeling dye PKH26-GL[33] (data not shown). In addition, PCR amplification of hepatic DNA prepared from rats transplanted with BAG2-WB cells demonstrated DNA sequences corresponding to the Tn5 neomycin resistance gene from the BAG2-retroviral construct,[32] as shown in Figure 3.

When transplanted subcutaneously, the tumor-derived GN6TF and GP7TB cells produce poorly differentiated neoplasms containing spindle-shaped cells that resemble mesenchymal cells morphologically (Figure 4, A and E). As shown in Table 1, these cell lines are highly aggressive subcutaneously, forming tumors of 0.5 to 1 cm diameter in 100% of the hosts after a short latency period (18 to 21 days for cells transplanted into neonatal animals and 28 days for cells transplanted into adult animals). Infection of the tumor cell lines with the BAG2-retrovirus altered neither their tumorigenicity nor latency after subcutaneous injection (Table 1), and the subcutaneous tumors resulting from BAG2-GN6TF and BAG2-GP7TB cells demon-

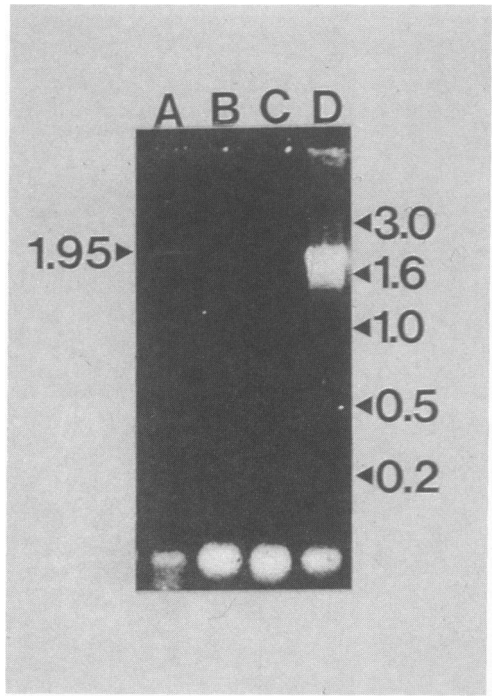

Figure 3. *PCR detection of BAG2-retroviral DNA in genomic DNA prepared from liver tissue of transplanted rats. Genomic DNA was prepared from representative samples of liver excised from normal rat liver, control rat liver transplanted with parental WB-F344 cell lines, or from the liver of a rat that had been transplanted with BAG2-WB cells. PCR reactions were carried out using 100 to 200 ng template DNA. PCR products were analyzed on 2% agarose gels containing 40 mmol/L Tris-acetate/1 mmol/L ethylenediaminetetraacetic acid (pH 7.6) and stained with ethidium bromide. The migration of DNA molecular size standards are indicated (kb). PCR reaction products from:* **lane A:** *DNA extracted from livers transplanted with BAG2-WB cells (41 days following transplantation);* **lane B:** *normal control liver DNA;* **lane C:** *control liver transplanted with parental WB-F344 cells; and* **lane D:** *control plasmid template containing the Tn5 neomycin resistance gene (from pSV2neo). Amplification of the neomycin resistance gene of the BAG2-retroviral DNA produces a 1.95-kb fragment using the primers described.*

strated intense blue staining when subjected to β-galactosidase histochemistry (Figure 4, A and E). When BAG2-GP7TB cells were transplanted into the liver, no alteration in tumorigenicity was observed (Table 1); small tumors (<0.3 cm diameter), which stained intensely blue by β-galactosidase histochemistry (Figure 4, F and G) and demonstrated bright orange fluorescence from PKH26-GL (Figure 4H), were evident in the liver within one week after transplantation. Transplanted BAG2-GP7TB cells formed expanding masses of similar cells within the liver; tumor cells were in contact with host hepatocytes only at the peripheries of the tumors. Unlike the spindle-shaped cells in subcutaneous tumors, liver tumors from the same cell line (BAG2-GP7TB) contained plump cells of epithelial morphology (Figure 4, F and G), suggesting that the influence of the hepatic microenvironment results in more highly differentiated tumors. Whereas BAG2-GP7TB tumors could be detected within the liver tissue shortly after transplantation, the hepatic tumors seemed to grow more slowly than did subcutaneous tumors. This suggestion is based on our observation that BAG2-GP7TB cells form large tumors (>1.0 cm diameter) at subcutaneous sites with an average latency of 21 days following the transplantation of 1×10^6 cells (Table 1), whereas the same cells formed small tumors (~0.3 cm diameter) in the liver in a comparable length of time following the injection of 5×10^6 cells.

In contrast to the results obtained with BAG2-GP7TB tumor cells, the BAG2-GN6TF tumor cells failed to produce tumors following transplantation of 5×10^6 cells into the liver. Instead, numerous β-galactosidase-positive cells morphologically resembling hepatocytes were individually integrated into hepatic plates (Figures 4, B and C), similar to the diploid BAG2-WB cells. These β-galactosidase-positive hepatocytelike cells were detected up to 87 days following transplantation, a period that is fourfold greater than the average latency period for tumor formation when 1×10^6 cells of the same line are transplanted subcutaneously (Table 1). Fluorescence microscopy of liver sections from animals transplanted with BAG2-GN6TF cells that had been fluorescently labeled with PKH26-GL dye also revealed the presence of cells showing specific orange fluorescence (Figure 4D).

Discussion

Recent studies have shown that differentiated hepatocytes isolated from enzymatically prepared suspensions of murine liver cells integrate into the hepatic plates following transplantation into livers of congenic animals.[43,44] Our observations show that WB-F344 rat liver epithelial cells also integrate into hepatic plates and morphologically differentiate into hepatocytes. Because the functionally and structurally simple WB-F344 liver epithelial cell line was isolated directly from the normal liver of a rat[21] under conditions that excluded mature hepatocytes,[7–9] our combined results provide evidence that these cells represent an hepatocytic stem cell that exists in normal adult rat liver. By the transplantation technique utilized in these studies, β-galactosidase-positive hepatocytelike cells were observed only in the hepatic plates among the host hepatocytes, suggesting that the microenvironment of the hepatic lobular parenchyma exerts a strong stimulus for differentiation of stem cells into hepatocytes. Studies aimed at characterizing the expression of

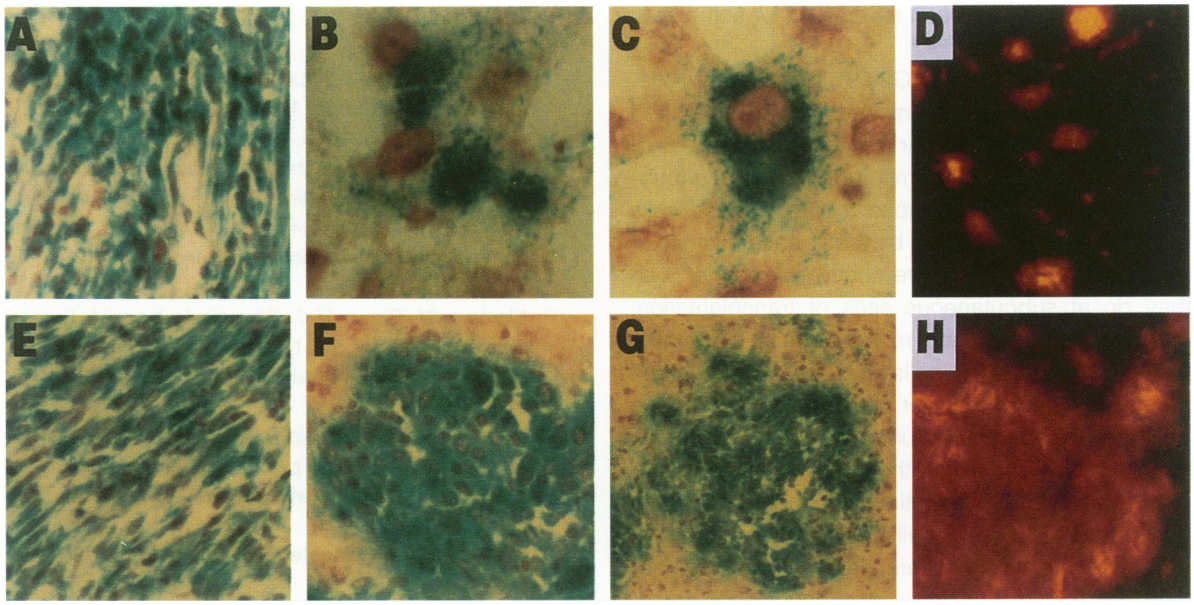

Figure 4. *Behavior of BAG2-GN6TF and BAG2-GP7TB cells following transplantation into different tissue environments. Cryosections from subcutaneous tumors or liver tissues were fixed and analyzed for the presence of transplanted cells.* **A,E:** *Cryosections from spindle-cell tumors arising with short latency (17 to 21 days) following subcutaneous transplantation of BAG2-GN6TF* **(A)** *and BAG2-GP7TB* **(E)** *into 1-day-old syngeneic host rats.* **B,C:** *BAG2-GN6TF cells integrated into hepatic plates and morphologically differentiated in the liver of an adult syngenic rat 87 days following transplantation without formation of tumors.* **D:** *BAG2-GN6TF cells incorporated into the hepatic plates of a host rat detected using PKH26-GL membrane labeling dye 30 days following transplantation.* **F** *to* **H:** *Transplantation of BAG2-GP7TB cells into the livers of adult syngeneic rats resulted in the formation of tumors that exhibited a higher degree of differentiation than those observed at subcutaneous sites. These tumors were visualized using both X-gal histochemistry* **(F, G)** *and PKH26-GL membrane labeling dye* **(H)**. *(Magnifications:* **A, D, E** *to* **H**, ~ ×300; **B, C**, ~ ×450).

hepatocyte-specific markers of differentiation in WB-F344 cells following intrahepatic transplantation, which will define the degree of functional differentiation accompanying the observed morphological differentiation of these cells in the liver, are currently in progress.

Because all the transplanted BAG2-WB cells were found to be located in the lobular parenchymal compartment, our studies do not answer the question of whether WB-F344 cells can differentiate along the bile duct cell lineage when the proper stimulus is provided. Bile duct cells form from the embryonic hepatoblast following an inductive stimulus provided by contact with the portal tract mesenchyme.[1] It is possible that transplanted BAG2-WB cells may require contact with the portal mesenchyme to produce bile ducts. Alternatively, the WB-F344 cells may have advanced along the hepatocyte differentiation pathway from true bipotential stem cells and may be able to differentiate only into

Table 1. *Transplantation Site-Specific Tumorigenicities of BAG2-GN6TF, BAG2-GP7TB, and Parental Cell Lines*

	Subcutaneous transplantation*			Transplantation to the liver†		
Cell line	Tumors per rats injected	Tumorigenicity (%)	Latency‡ (days)	Tumors per rats injected	Tumorigenicity (%)	Latency§ (days)
BAG2-GN6TF	21/21	100	21 ± 4	0/3	0	N.A.‖
BAG2-GP7TB	25/25	100	21 ± 3	9/9	100	19 ± 3
GN6TF	7/7 (5/5)¶	100	21 ± 1	0/5	0	N.A.
GP7TB	8/8 (3/3)¶	100	18 ± 2	5/5	100	13 ± 5

* Cell lines were transplanted subcutaneously (1×10^6 cells injected) into 1-day-old Fischer 344 rat pups. Subcutaneous tumors were harvested after reaching a size of approximately 1 cm diameter.

† Cell lines were transplanted intrahepatically (5×10^6 cells injected) into young adult Fischer 344 rats by direct injection into the median liver lobe or dorsal tip of the spleen. Animals were sacrificed at various intervals following transplantation and livers inspected histochemically for the presence of tumor masses.

‡ Mean time to the harvest of approximately 1 cm diameter tumors.

§ Rats subjected to liver transplantation with tumor cell lines were sacrificed at regular intervals from 7 to 30 days following transplantation. Thus, this value represents the time required for tumors to become macroscopically detectable.

‖ Not applicable.

¶ GN6TF and GP7TB cells were also evaluated for tumorigenicity following subcutaneous transplantation (1×10^7 cells injected) into adult Fischer 344 rats (approximately 200 g). Average latency for subcutaneous tumor formation in adult animals was 28 ± 1 day for each of these cell lines.

hepatocytes, as has been suggested for some lines of cultured oval cells.[45,46]

BAG2-GN6TF and BAG2-GP7TB cells are derived from aneuploid parental cell lines (GN6TF and GP7TB)[27] that carry similar marker chromosomes (S Steadman, LW Lee, GJ Smith, JW Grisham, unpublished observations). Despite this fact, these cells are differentially regulated by the hepatic microenvironment to grow in a more controlled manner than they do at subcutaneous sites of transplantation. The apparent complete morphological differentiation of BAG2-GN6TF cells suggests that the microenvironment of the liver not only suppresses the ability of this particular tumor cell line to form tumors but also stimulates them to integrate into hepatic plates and differentiate into hepatocytes. In contrast, BAG2-GP7TB cells form tumors in the liver that are more highly differentiated morphologically than are tumors that form at subcutaneous transplantation sites, suggesting that the regulatory influence of the liver parenchyma can induce partial reversion of the transformed phenotype without complete suppression of tumorigenicity. These observations suggest that transformed, highly tumorigenic, rat liver epithelial stemlike cells retain part or all of the cellular targets (cell-surface receptors, cell adhesion proteins, etc.) that mediate the cellular response to microenvironmental influences within the liver. The effects of the lobular parenchymal microenvironment on these transformed rat liver epithelial stemlike cells may require the incorporation of the transplanted cells into the hepatic plates and the formation of intimate cell-cell contacts with differentiated hepatocytes, contact or interaction with the liver extracellular matrix, and/or the presence of specific transactivating factors produced by cells of the normal liver. The present studies have not determined whether the suppressive effects of the lobular parenchyma represent a permanent genetic reprogramming of the transformed cells, resulting in a more differentiated cell phenotype with lower capacity for proliferation, or a tissue-specific and reversible cis-suppression that is dependent upon factors present within the adult liver for the maintenance of suppression.

Acknowledgments

We thank Drs. William K. Kaufmann and Gregory Tsongalis for reading a draft of the manuscript and Ms. Theo Cantwell for editing it.

References

1. Wilson JW, Groat CS, Leduc EH: Histogenesis of the liver. Ann NY Acad Sci 1963, 111:8–24
2. Germain L, Blouin M-J, Marceau N: Biliary epithelial and hepatocytic cell lineage relationships in embryonic rat liver as determined by the differential expression of cytokeratins, α-fetoprotein, albumin, and cell surface-exposed components. Cancer Res 1988, 48: 4909–4918
3. Marceau N, Blouin M-J, Germain L, Noel M: Role of different epithelial cell types in liver ontogenesis, regeneration and neoplasia. In Vitro Cell Dev Biol 1989, 25:336–341
4. Grisham JW: A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver autoradiography with thymidine-H^3. Cancer Res 1962, 22:842–849
5. Sell S: Is there a liver stem cell? Cancer Res 1990, 50: 3811–3815
6. Aterman K: The stem cells of the liver—a selective review. J Cancer Res Clin Oncol 1992, 118:87–115
7. Grisham JW, Thal SB, Nagel A: Cellular derivation of continuously cultured epithelial cells from normal rat liver. Gene Expression and Carcinogenesis in Cultured Liver. Edited by Gerschenson LE, Thompson EB, New York, Academic Press, 1974, pp 1–23
8. Grisham JW: Cell types in long-term propagable cultures of rat liver. Ann NY Acad Sci 1980, 349:128–137
9. Furukawa K, Shimada T, England P, Mochizuki Y, Williams GM: Enrichment and characterization of clonogenic epithelial cells from adult rat liver and initiation of epithelial cell strains. In Vitro Cell Dev Biol 1987, 23: 339–348
10. Farber E: Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene, and 3′-methyl-4-dimethyl aminobenzene. Cancer Res 1956, 16:142–155
11. Grisham JW, Hartroft WS: Morphologic identification by electron microscopy of "oval" cells in experimental hepatic degeneration. Lab Invest 1961, 10: 317–332
12. Lombardi B: On the nature, properties and significance of oval cells. Recent Trends in Chemical Carcinogenesis. Edited by Pani P, Feo F, Columbano A, Cagliari ESA, 1982, vol. I, pp 36–52
13. Sell S, Leffert HL: An evaluation of the lineages in the pathogenesis of experimental hepatocellular carcinoma. Hepatology 1982, 2:77–86
14. Sirica AE, Mathis GA, Sano N, Elmore LW: Isolation, culture, and transplantation of intrahepatic biliary epithelial cells and oval cells. Pathobiology 1990, 58: 44–64
15. Ogawa K, Minase T, Onoe T: Demonstration of glucose 6-phosphatase activity in the oval cells of rat liver and the significance of the oval cells in azo dye carcinogenesis. Cancer Res 1974, 34:3379–3386

16. Sell S, Salmon J: Light- and electron-microscopic autoradiographic analysis of proliferating cells during the early stages of chemical hepatocarcinogenesis in the rat induced by feeding N-2-fluorenylacetamide. Am J Pathol 1984, 114:287–300
17. Evarts RP, Nagy P, Thorgeirsson SS: A precursor-product relationship exists between oval cells and hepatocytes in rat liver. Carcinogenesis 1987, 8:1737–1740
18. Germain L, Noel M, Gourdeau H, Marceau N: Promotion of growth and differentiation of rat ductular oval cells in primary culture. Cancer Res 1988, 48: 368–378
19. Evarts RP, Nagy P, Nakatsukasa H, Marsden E, Thorgeirsson SS: *In vivo* differentiation of rat liver oval cells into hepatocytes. Cancer Res 1989 49:1541–1547
20. Lemire JM, Shiojiri N, Fausto N: Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine. Am J Pathol 1991, 139: 535–552
21. Tsao M-S, Smith JD, Nelson KG, Grisham JW: A diploid epithelial cell line from normal adult rat liver with phenotypic properties of oval cells. Exp Cell Res 1984, 154:38–52
22. Tsao M-S, Grisham JW, Nelson KG, Smith JD: Phenotypic and karyotypic changes induced in cultured rat hepatic epithelial cells that express the "oval" cell phenotype by exposure to N-methyl-N'-nitro-N-nitrosoguanidine. Am J Pathol 1985, 118:306–315
23. Tsao M-S, Grisham JW, Chou BB, Smith JD: Clonal isolation of populations of γ-glutamyl transpeptidase-positive and -negative cells from rat liver epithelial cells chemically transformed *in vitro*. Cancer Res 1985, 45:5134–5138
24. Tsao M-S, Grisham JW, Nelson KG: Clonal analysis of tumorigenicity and paratumorigenic phenotypes in rat liver epithelial cells chemically transformed *in vitro*. Cancer Res 1985, 45:5139–5144
25. Grisham JW, Tsao M-S, Lee DC, Earp HS: Sequential changes in the epidermal growth factor receptor/ligand function in cultured liver epithelial cells transformed chemically *in vitro*. Pathobiology 1990, 58: 3–14
26. Tsao M-S, Grisham JW: Hepatocarcinomas, cholangiocarcinomas, and hepatoblastomas produced by chemically transformed cultured rat liver epithelial cells. Am J Pathol 1987, 127:168–181
27. Tsao M-S, Grisham JW: Phenotypic modulation during tumorigenesis by clones of transformed rat liver epithelial cells. Cancer Res 1987, 47:1282–1286
28. Lee LW, Raymond VW, Tsao M-S, Lee DC, Earp HS, Grisham JW: Clonal cosegregation of tumorigenicity with overexpression of c-myc and transforming growth factor α genes in chemically transformed rat liver epithelial cells. Cancer Res 1991, 51:5238–5244
29. Richter A, Sanford KK, Evans VJ: Influence of oxygen and culture media on plating efficiency of some mammalian tissue cells. J Natl Cancer Inst 1972, 49: 1705–1712
30. Mann R, Mulligan RC, Baltimore D: Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 1983, 33:153–159
31. Cepko CL, Roberts BE, Mulligan RC: Construction and applications of a highly transmissable murine retrovirus shuttle vector. Cell 1984, 37:1053–1062
32. Price J, Turner D, Cepko C: Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci USA 1987, 84:156–160
33. Horan PK, Slezak SE: Stable cell membrane labelling. Nature 1989, 340:167–168
34. Higgins GM, Anderson RM: Experimental pathology of the liver: I. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 1931, 12: 186–202
35. Sanes JR, Rubenstein JLR, Nicolas J-F: Use of recombinant retrovirus to study the postimplantation cell lineage in mouse embryos. EMBO J 1986, 5:3133–3142
36. Wallenfels K, Weil R: β-galactosidase. The Enzymes, vol. VII. Edited by Boyer PD, New York, Academic Press, 1972, pp 617–663
37. Hiraiwa M, Uda Y: GM1 ganglioside β-galactosidases from bovine liver. Jpn J Exp Med 1988, 58:129–138
38. Rodriguez-Berrocal FJ, de la Cadena MP, Cabezas JA, Perez-Gonzalez N: Purification and characterization of acid β-D-galactosidase from rabbit spleen. J Biochem 1988, 104:66–71
39. Martinez-Zorzano VS, Rodriguez-Berrocal FJ, Cabezas JA, de la Cadena MP: Comparative studies of two acid β-galactosidases from rabbit and bovine kidney. Kidney Int 1989, 35:1295–1299
40. Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H: Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 1982, 19:327–336
41. Fiers W, Contreras R, Haegeman G, Rogiers R, Van de Voorde A, Van Heuverswyn H, Van Herreweghe J, Volckaert G, Ysebaert M: Complete nucleotide sequence of SV40 DNA. Nature 1978, 273:113–120
42. Dannenberg A, Suga M: Histochemical stains for macrophages in cell smears and tissue sections: β-galactosidase, acid phosphatase, nonspecific esterase, succinic dehydrogenase, and cytochrome oxidase. Methods for Studying Mononuclear Phagocytes. Edited by Adams DO, Edelson PJ, Koren HS, New York, Academic Press, 1981, pp 375–396
43. Ponder KP, Gupta S, Leland F, Darlington G, Finegold M, DeMayo J, Ledley FD, Chowdhury JR, Woo SLC: Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. Proc Natl Acad Sci USA 1991, 88:1217–1221
44. Gupta S, Aragona E, Vemuru RP, Bhargava KK, Burk RD, Chowdhury JR: Permanent engraftment and function of hepatocytes delivered to the liver: implications

for gene therapy and liver repopulation. Hepatology 1991, 14:144–149

45. Goyette M, Faris R, Braun L, Hixson D, Fausto N: Expression of hepatocyte and oval cell antigens in hepatocellular carcinomas produced by oncogene-transfected liver epithelial cells. Cancer Res 1990, 50: 4809–4817
46. Shiojiri N, Lemire JM, Fausto N: Cell lineages and oval cell progenitors in rat liver development. Cancer Res 1991, 51:2611–2620